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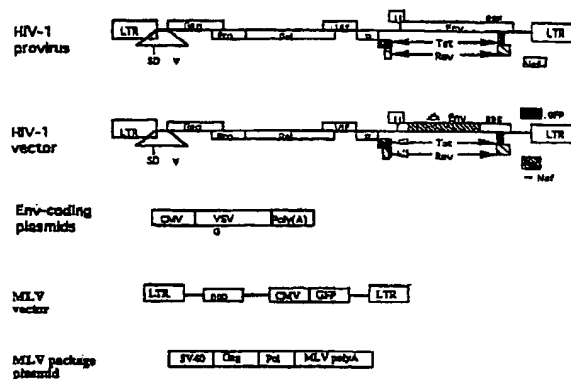
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(54) Title: USE OF LENTIVIRAL VECTORS FOR ANTIGEN PRESENTATION IN DENDRITIC CELLS



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(57) Abstract: The present invention relates to methods of inducing an immune response against an antigen in a subject by administering to the subject, a therapeutically effective amount of a dendritic cell, or of a progenitor thereof, which is transduced with a replication defective pseudotyped lentiviral vector containing a nucleic acid sequence encoding a polypeptide, which is expressed in said dendritic cell and is presented on the surface of the dendritic cell, such that, following administration to a subject, an immune response is induced against the antigen in the subject. The invention also relates to a method of inducing an immune response against an antigen in a subject, by transducing a dendritic cell, or a progenitor of a progenitor thereof, with a pseudotyped lentiviral vector containing a nucleic acid sequence encoding polypeptide, which is expressed in the dendritic cell and is presented on the surface of the dendritic cell, and contacting the dendritic cell with a T cell to produce an activated T cell. The invention further provides a method of activating a T cell by contacting a T cell with a dendritic cell, or a progenitor of a dendritic cell, which expresses an antigen encoded by a transduced pseudotyped lentiviral vector, on its surface. The invention also relates to a vector, which contains an envelope protein from a virus other than a lentivirus, and a lentivirus genome encoding non-functional env and nef gene products; or a lentivirus genome encoding env and nef gene products and at least three accessory protein gene products, for example, non-functional vif, vpr and vpu.



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USE OF LENTIVIRAL VECTORS FOR ANTIGEN PRESENTATION IN DENDRITIC CELLS

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5

FIELD OF THE INVENTION

The present invention relates generally to the field of immunology and induction of immune responses and more specifically to lentiviral vectors and the use of such vectors, and of dendritic cells transduced with the vectors, to deliver an antigenic epitope for inducing immunity.

10

BACKGROUND OF THE INVENTION

The host immune system provides a sophisticated defense mechanism that enables the recognition and elimination of foreign entities such as infectious agents or neoplasms from the body. When functioning properly, an effective immune system distinguishes between foreign invaders and the host's own tissues. The ability to specifically ignore the host's own tissues is referred to as immune tolerance. Immune tolerance to self normally develops at birth, when self antigens are brought to the thymus by antigen presenting cells (APCs). APCs play a crucial role in "programming" the immune system by specifically indicating which antigens are considered foreign and, therefore, are targeted by the immune system.

20

Dendritic cells (DCs) are efficient APCs that initiate immune response to peptide antigens associated with class I and class II MHC molecules (Freudenthal and Steinman, *Proc. Natl. Acad. Sci. USA* 87:7698, 1990; Steinman, *Ann. Rev. Immunol.* 25 9:271, 1991). DCs represent a small subpopulation of widely distributed bone marrow derived leukocytes that are the only natural APCs able to prime naive T cells. DCs activate both CD4+ and CD8+ T lymphocyte primary immune responses and are at least as effective as other APCs such as monocytes in stimulating secondary immune

responses (Peters et al., *Immunol. Today* 17:273, 1997). In lymphoid tissues, the DCs are primarily localized in the T cell areas. The B cell areas or follicles of lymphoid organs contain a second type of DC, the follicular dendritic cell (FDC).

5 Several populations of human DCs have been identified in peripheral blood, including the myeloid DCs, which can be produced from precursors after *in vitro* culture with granulocyte-monocyte colony stimulating factor (GM-CSF) and interleukin-4 (IL4). The cytokine IL4 appears to be necessary to inhibit emergence of monocytes/macrophages. Functionally and phenotypically mature DCs were
10 identified among other cell types after expansion of proliferative CD34+ progenitors in GM-CSF and TNF α . Large numbers of fully functional DCs have been generated from purified adherent monocytes (Mo-DC) cultured in GM-CSF and IL-4 (Kan-Mitchell et al., In: *Leukocyte Typing VI*, T. Kishimoto et al., New York, 1997). Murine leukemia virus (MLV) based vectors have been used to transduce CD34+
15 hematopoietic progenitor cells, which then were differentiated into DCs after weeks of *in vitro* culture. These DCs were able to generated a specific T cell mediated antitumor immune response *in vitro* (Henderson et al., *Cancer Res.* 56:3763, 1996; Reeves et al., *Cancer Res.* 56:5672 1996), although their relationship to naturally occurring DC is unknown.

20 Recent evidence suggests that DCs are potent physiological adjuvants for induction of prophylactic or therapeutic antitumor immunity. In mice, DCs pulsed with short synthetic peptides *in vitro* elicited protective immunity mediated by tumor specific CD4+ helper T cells or CD8+ cytotoxic T cells *in vivo* (Nair et al., *Int. J.*
25 *Cancer* 70:706, 1977). Therapeutic efficacy was suggested by results of a pilot study, in which lymphoma patients treated with autologous DCs from the blood pulsed *ex vivo* with the lymphoma patients' idiotype produced antibodies and experienced clinical responses (Lynch et al., *Nature Med.* 3:625, 1997).

30 Although recent developments in combination drug therapy have had a tremendous impact on the treatment of AIDS patients in developed countries, the AIDS epidemic continues apace in its global devastation. The most effective means to curtail the spread of this disease would be to develop a safe and efficacious vaccine. However, a major problem in AIDS vaccine development is that only a
35 weak and transient immune response is induced by currently available vaccines.

There is compelling evidence from patient studies that HIV specific cytotoxic T lymphocytes (CTLs) are central to controlling HIV infection (Rowland-Jones et al., *Adv. Immunol.* 65:277,1997). Strong CTL responses have been identified particularly in nonprogressive patients and at the sites of infection. CTLs also inhibit virus replication *in vitro*, and react to most HIV gene products, particularly to the *gag*, *pol*, and *env* gene products; this reactivity has been mapped. CTL epitopes cluster together in regions of *pol*, but were more evenly distributed through *gag*. Most epitopes were identified based on the binding motif of the class I antigen (Brander et al., *Clin. Exp. Immunol.* 101:107, 1995). HLA-A2 donors recognized at least three epitopes on *gag* and two on *pol*, one of which is an immunodominant epitope in the active site of the reverse transcriptase. Spontaneous response to *pol*, which should be a valuable target for immunotherapy, was rarely observed (McMichael and Walker, *AIDS* 8 (suppl. IZ): S155, 1994; Goulder et al., *Nature Med.* 3:212, 1997). Thus, a need exists to develop vaccines that can stimulate an effective immune response against HIV. The present invention satisfies this need and provides additional advantages.

SUMMARY OF THE INVENTION

The present invention relates to methods of inducing an immune response against an antigen in a subject by administering to the subject, a therapeutically effective amount of a dendritic cell, or of a progenitor thereof, which is transduced with a replication defective pseudotyped lentiviral vector encoding nonfunctional *nef* and *env* gene products and, if desired, three or more nonfunctional accessory gene products. The vector contains, at least in part, a nucleic acid sequence encoding an antigen, which is expressed in said dendritic cell and is presented on the surface of the dendritic cell, such that, following administration to a subject, an immune response is induced against the antigen in the subject. The dendritic cell can be, for example, an immature dendritic cell such as a Langerhans cell, which is present in the skin. The DC can be a non-dividing dendritic cell. A progenitor of a dendritic cell can be a CD34⁺ cell, for example.

A pseudotyped lentiviral vector of the invention can contain a nucleic acid sequence encoding an expressible polypeptide, for example, a cytokine such as an interleukin, Flt-3/Flk-2 ligand, granulocyte macrophage colony stimulating factor, stem cell factor, or the like; or a tumor antigen; and, if desired, can encode more than one antigen, including a fusion polypeptide containing more than one antigen. The

encoded polypeptide antigen also can be a lentiviral antigen or a fragment thereof, for example, a human immunodeficiency virus-1 (HIV-1) antigen such as gag, pol, env, vpr, vif, nef, vpx, tat, rev, or vpu.

5 A pseudotyped lentiviral vector of the invention contains an envelope protein from a different virus than a virus from which the vector nucleic acid is obtained, including a lentivirus or a virus other than a lentivirus. For example, the envelope can be a vesicular stomatitis virus G (VSV-G) env protein or a Moloney leukemia virus (MLV) env protein. The vector nucleic acid can be obtained, for example, from
10 HIV-1, thus providing a pseudotyped HIV-1 vector, the genome of which encodes nonfunctional HIV-1 env and nef gene products, and, if desired, nonfunctional accessory gene products, for example, nonfunctional HIV-1 env, nef, vif, vpr, or vpu gene products. The pseudotyped lentiviral vector, for example, a pseudotyped HIV-1 vector, also can contain an expressible nucleotide sequence that is heterologous to the
15 particular lentivirus.

 The invention also relates to a method of inducing an immune response against an antigen in a subject, by transducing a dendritic cell, or a progenitor of a DC, with a pseudotyped lentiviral vector. The vector can include a nucleic acid
20 sequence encoding an antigen, which is expressed in the dendritic cell and is presented on the surface of the dendritic cell. The transduced dendritic cell is contacted with a T cell to produce an activated T cell. Either the pseudotyped lentiviral vector, the transduced dendritic cells, or progenitor thereof; or the activated T cells are administered to the subject. The dendritic cell can be transduced *in vivo* or
25 *in vitro*. When the transducing is performed *in vivo*, the vector can be administered intradermally, for example, such that dendritic cells in the skin, generally Langerhans cells, are transduced. When the transducing is performed *in vitro*, the transduced dendritic cell is contacted with the T cell *in vitro* or *in vivo*. When the contacting is performed *in vitro*, the activated T cells are administered to the subject.

30 The invention further provides a method of activating a T cell by contacting the T cell with a dendritic cell, or a progenitor of a dendritic cell. The dendritic cell, or progenitor thereof, expresses an antigen, which is encoded by a transduced pseudotyped lentiviral vector, on its surface. Examples of dendritic cells include
35 immature dendritic cells such as a Langerhans cells; non-dividing dendritic cells; or CD34⁺ cells. The activating can occur in a subject *in vivo*, for example, by

administering a transduced dendritic cell, or a progenitor thereof, to the subject intradermally; or by transducing the dendritic cell *in situ* with the vector. The activating also can occur *in vitro*.

5 The invention also relates to a vector that contains an envelope protein from a virus other than a lentivirus and contains a lentivirus genome encoding nonfunctional env and nef gene products. The envelope protein can be from any lentivirus, provided it is from a lentivirus that is different from that providing the vector nucleic acid, or can be from an enveloped virus other than a lentivirus, for example, a VSV-G env
10 protein or a Moloney leukemia virus env protein. In one embodiment, the vector nucleic acid is from an HIV-1 genome, and the env protein is a VSV-G env protein. The vector can encode an HIV-1 antigen, or can contain a heterologous nucleic acid sequence encoding any desired polypeptide.

15 The invention further provides a vector that contains an envelope protein from a virus other than a lentivirus and contains a lentivirus genome encoding nonfunctional env and nef gene products, and nonfunctional accessory protein gene products. In one embodiment, the lentiviral vector is a pseudotyped lentiviral vector having a VSV-G env protein. In another embodiment, the lentivirus genome is an
20 HIV-1 genome having deletions of nucleic acid sequences encoding HIV-1 nef, env and at least three accessory proteins such as vif, vpr and vpu. If desired, the vector can contain a heterologous nucleic acid sequence, which can encode an antigen.

BRIEF DESCRIPTION OF THE DRAWINGS

25 FIG. 1 is a schematic illustration of an HIV-1 provirus, and env deleted HIV-1 vector encoding green fluorescent protein (GFP), Env encoding plasmids, a murine leukemia virus (MLV) vector encoding GFP, and a MLV package plasmid.

30 FIG. 2 is a plot of two color flow cytometric analysis of the expression of GFP in CD34+ cells delivered by an HIV-1 vector.

DETAILED DESCRIPTION OF THE INVENTION

35 The present invention provides, in general, a dendritic cell (DC) based vaccine and a strategy to induce virus specific cytotoxic T lymphocyte (CTL) immunity. As such, the invention provides a method of inducing an immune response in a subject by

administering to the subject a therapeutically effective amount of a DC, or of a progenitor thereof, transduced with a replication defective pseudotyped lentiviral vector having a nucleic acid sequence encoding an antigen, such that the antigen is presented on the surface of the dendritic cell.

5

Also provided is a method of inducing an immune response in a subject by transducing a DC, or a progenitor of a DC with a pseudotyped lentiviral vector, which contains a nucleic acid sequence encoding an antigen, such that the antigen is presented on the surface of the DC, then contacting the transduced DC with a T cell to produce an activated T cell; at least one of the pseudotyped lentiviral vector, the transduced DC or the T cell is administered to the subject. In addition, a method of activating a T cell by contacting the T cell with a DC having an antigen on its surface is provided. In this method, the DC contains a pseudotyped lentiviral vector having a nucleic acid sequence encoding the antigen, which can be an antigen encoded by the lentivirus, or can be an antigen encoded by a heterologous nucleic acid sequence present in the vector. Contacting the T cell with the DC results in activation of the T cell.

Vectors of the invention contain a lentiviral genome which has been modified such that the nef and env gene products and, if desired, accessory protein gene products such as vif, vpr, vpu, tat, rev or others, either are not produced or are produced in a nonfunctional form, for example, truncated. As used herein, the term "nonfunctional," when used in reference to a viral gene product, means that the gene product does not have the function it normally would have when expressed from a virus in which it naturally is expressed. The term "nonfunctional gene product" is used broadly herein to include an expressed defective gene product, for example, a mutated or truncated gene product, as well as to the complete absence of the gene product due, for example, to deletion of the nucleic acid sequence encoding the gene product or to a mutation of a regulatory element from which expression of the gene product depends. For purposes of the present invention, reference to a "nonfunctional gene product" is made in comparison to the corresponding gene product that normally is expressed from the genome of the virus contributing the vector genome. Thus, while a nonfunctional gene product does not have the function normally associated with it, it can be useful, for example, as an antigen.

35

A nucleic acid sequence encoding a lentiviral protein such as an HIV-1 env protein can be deleted completely, or in part, such that no gene product is expressed or a nonfunctional portion of the gene product is expressed. A nucleic acid sequence also can encode a nonfunctional gene product due, for example to a substitution or insertion of one or more nucleotides in the sequence encoding the gene product, such that either 1) there is no transcription or translation of the sequence and, therefore, no gene product produced; or, 2) if transcription and translation of the sequence occurs, a nonfunctional peptide or polypeptide is produced. For example, a deletion, substitution or insertion of one or more nucleotides into a nucleic acid sequence encoding a gene product can introduce a STOP codon into the sequence, such that a truncated nonfunctional gene product is produced.

It should be recognized that the nonfunctional gene products of a particular viral vector of the invention can be nonfunctional for various reasons. For example, one or more of the gene products may be nonfunctional due to deletion of all or part of the nucleic acid sequences encoding the gene products; one or more other gene products may be nonfunctional due to the incorporation of a STOP codon in the encoding nucleic acid sequences; and still another gene product may be nonfunctional due to a directed mutation in a regulatory element required for its expression. These and other methods known in the art are useful for rendering selected gene products nonfunctional in a viral vector of the invention, and one or more of the methods can be used in constructing a single viral vector of the invention.

Reference is made herein to various forms of a virus or viral nucleic acid molecules. In general, the terms "viral vector," "viral vector genome" and "vector nucleic acid sequence" refer to a nucleic acid molecule of the invention (vector) contained in a viral particle and is introduced into a cell by transduction. The term "viral genome" refers to the nucleic acid molecule that normally is present in a naturally occurring virus. The term "viral particle," "viral virion" or "vector virion" refers to an infectious agent, which contains a viral coat and a viral genome or a vector genome. Unless otherwise specified, the term "vector," when used in reference to a composition of the invention, is used broadly to refer to a viral vector or a vector virion of the invention.

Vectors of the invention are exemplified herein by pseudotyped HIV-1 vectors, which contain an HIV-1 viral genome having a deletion of the nucleic acid

sequences encoding nef and env proteins. In addition, pseudotyped HIV-1 vectors containing an HIV-1 viral genome having deletions of the nef, env, vif, vpr and vpu genes are exemplified. In particular, VSV-G pseudotyped HIV-1 vectors are exemplified herein. A vector of the invention is useful, for example, for inducing
5 anti-HIV-1 immunity in an individual, or for inducing immunity to an antigen encoded by a nucleic acid sequence contained in the vector.

As disclosed herein, an immune response can be induced in a subject by administering to the subject a DC, or progenitor thereof, which has been transduced
10 with a pseudotyped lentiviral vector containing a nucleic acid sequence of interest such that the nucleic acid sequence of interest is expressed in the DC. In one example, HIV antigens were stably introduced into human DCs using HIV-1 vectors pseudotyped with the VSV-G protein, which allows highly efficient transduction into the CD34+ progenitor cells as well as adherent monocytes (Mo-DCs). The HIV-1
15 vectors encoding HIV-1 antigens and a reporter gene successfully transduced CD34+ cells and Mo-DCs with high efficiency relative to murine retroviral vectors. Thus, the vectors of the invention provide substantial advantages over vectors such as those described in U.S. Patent No. 5,851,813, in part, because the vectors of the invention are pseudotyped and, therefore, can be designed to infect a substantially broader range
20 of target host cells.

As used herein, the term "dendritic cell" has its well understood meaning of a bone marrow derived leukocyte that acts as an antigen presenting cell (APC), and can prime naive T cells. *In vivo*, DCs present antigens to and activate naive CD4+ T cells
25 (Levin et al., *J. Immunol.* 151: 6742-6750, 1993). Several populations of human DCs have been identified, including myeloid DCs, which can be produced from precursors after *in vitro* culture with granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL4). DCs can be generated from highly purified adherent Mo-DCs cultured in GM-CSF and IL-4 (Kan-Mitchell et al., *supra*, 1997). Another
30 form of DCs are low density APCs (LDCs), which are found in fresh mobilized peripheral blood monocyte cells (PBMCs) that appear to function as mature APC, including in an allogeneic mixed lymphocyte reaction (MLR). Fresh LDCs express low levels of the monocyte marker CD24, and high levels of HLA-DR, and costimulatory molecules CD40, CD80, and CD86. In addition, Langerhans cells are
35 immature DCs present in the skin of mammals, including humans. As disclosed

herein, freshly isolated DCs, primary cultures of DCs, or DC cell lines can be utilized in the methods of the invention, or DC cells can be transduced *in situ*.

DCs useful in a method of the invention can be xenogeneic, allogeneic,
5 syngeneic or autologous with respect to the subject to which they are administered. Steinberg et al. (WO 93/20185) have disclosed methods for isolating primary DCs and their precursors from tissue. Granucci et al., WO 94/28113, and Paglia et al. (*J. Exp. Med.* 178:1893-1901, 1993) have disclosed DC cell lines isolated from primary cultures and immortalized. McKay et al. (U.S. Patent 5,648,219) have described
10 immortalized DC cell lines. Each of the references cited in this application is incorporated herein by reference.

DCs can be dividing or nondividing DCs. As used herein, the term
"non-dividing" refers to a cell that does not proceed through mitosis. Non-dividing
15 cells may be blocked at any point in the cell cycle, including G₀/G₁, G₁/S, and G₂/M and, therefore, are not actively dividing. Primary cultures of autologous DCs can be particularly useful for performing the *in vitro* methods of the invention.

As used herein, the term "DC progenitor" means a cell that can give rise to a
20 DC following appropriate signaling. DC progenitors, which express CD34 antigen, can be obtained using well known methods for purifying CD34⁺ cells (see, for example, Lane et al., *Blood* 85:275, 1985). An "immature dendritic cell" is a DC that expresses low levels of MHC class II molecules, but is capable of endocytosing antigenic proteins and processing them for presentation in a complex with MHC
25 class II molecules. Such immature DCs, which include Langerhans cells of the skin, can be stimulated to become activated DCs. An "activated DC" is a more mature DC that expresses class I and high levels of class II MHC molecules, adhesion molecules such as ICAM-1, and costimulatory molecules such as B7-2. An activated DC can endocytose antigenic peptides and process them for presentation.

30 The DCs can be obtained as a substantially enriched population of DCs. As used herein, the term "substantially enriched," when used in reference to a population of DCs or APCs, means a relatively homogeneous population of APCs that are relatively free from other cells with which they are naturally associated *in vivo*. In
35 general, a substantially enriched population of APCs is a population wherein the majority, or at least about 90%, of the cells, are the selected cell type. Thus, a

substantially enriched population of DCs contains about 10% or less fibroblasts or other immune cells and, generally, contains about 5% or less of such cells. A substantially enriched population of APCs can be obtained using well known and routine methods, including, for example, immunoaffinity chromatography using
5 monoclonal antibodies that specific for a determinant expressed only on population of DCs that is desired.

A substantially enriched population of DCs also can be obtained from mixed cell suspensions by positive selection (collecting only DCs), or negative selection
10 (removing cells that are not DCs). Methods for capturing such specific cells on affinity materials are well known and described, for example, by Wigzed et al., *J. Exp. Med.* 129:23, 1969; Wysocki et al., *Proc. Natl. Acad. Sci. USA* 75:2844, 1978; Schrempf-Decker et al., *J. Immunol. Meth.* 32:285, 1980; and Muller-Sieberg et al., *Cell* 44:653, 1986. Monoclonal antibodies specific for antigens expressed by mature,
15 differentiated cells have been used in a variety of negative selection strategies to remove undesired cells, for example to deplete T cells or malignant cells from allogeneic or autologous marrow grafts, respectively (Gee et al., *J. N.C.I.* 80:154, 1988). Purification of human hematopoietic cells by negative selection with monoclonal antibodies and immunomagnetic microspheres can be accomplished using
20 multiple monoclonal antibodies (Griffin et al., *Blood* 63:904, 1984). A substantially enriched DC population can be obtained from a mixture of lymphocytes because dendritic cells lack surface immunoglobulins (e.g., IgG) and T cell markers, and do not respond to B cell or T cell mitogens *in vitro*. DCs also fail to react with MAC-1 monoclonal antibody, which reacts with all macrophages. Therefore, MAC-1
25 provides a means of negative selection that can be used in order to produce a substantially enriched population of DC.

Useful procedures for separating and purifying cells include magnetic separation methods using antibody coated magnetic beads; affinity chromatography;
30 cytotoxic agents linked to a monoclonal antibody or used in conjunction with a monoclonal antibody, for example, and complement and cytotoxins; "panning" with antibodies attached to a solid matrix such as a plastic plate; or any other convenient method. Accurate and efficient separation of particular cell types such as immature DCs can be performed using, for example, fluorescence activated cell sorters (FACS),
35 which can have a plurality of color channels, low angle, and obtuse light scattering

detecting channels, and impedance channels, to facilitate detection and separation of the desired cell type.

5 In a method of the invention, DCs or progenitors of DCs are transduced with an effective amount of a pseudotyped lentiviral vector containing a nucleic acid sequence encoding a polypeptide, generally, a desired antigen. The nucleic acid sequence encoding the antigen can be transcribed and translated in the DC to produce the antigen, which can be processed and presented on the surface of the DC.

10 The vectors of the invention contain a nucleic acid sequence of a lentivirus genome and, therefore, are referred to generally as lentiviral vectors. Lentiviruses are retroviruses, which contain a viral genome consisting of RNA. As used herein, the term "lentivirus" refers to a genus of viruses that contain reverse transcriptase.

15 When a host cell is infected with a retrovirus, the genomic RNA is reverse transcribed into a DNA intermediate, which is integrated very efficiently into the chromosomal DNA of infected cells. The integrated DNA intermediate is referred to as a provirus.

20 The lentiviral vectors of the invention are recombinant lentiviruses, particularly recombinant immunodeficiency disease viruses, including human immunodeficiency virus (HIV), feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV), and the like. The recombinant lentiviral vectors are replication defective and, therefore, cannot assemble into infectious virions unless they are propagated in the presence of an appropriate helper nucleic acid sequence, for
25 example, in a helper virus containing cell line, in which appropriate sequences enabling encapsidation are expressed.

A recombinant lentivirus can be produced in a helper cell line that provides the missing viral functions. The helper cell line can contain a plasmid that
30 complements the nucleotide sequence missing in the recombinant vector and enables, for example, the packaging mechanism to recognize an RNA transcript for encapsidation. Helper cell lines which have deletions of the packaging signal include, but are not limited to, Ψ 2, PA317 and PA12. Suitable cell lines produce empty virions, since no genome is packaged. If a replication defective retroviral vector is
35 introduced into such helper cells, in which the packaging signal is intact, but the

structural genes are replaced by other genes of interest, the vector can be packaged and vector virion produced.

5 A retroviral genome, including a lentiviral genome, and the proviral DNA have three genes *gag*, *pol*, and *env*, which are flanked by two long terminal repeat (LTR) sequences. The *gag* gene encodes the internal structural (matrix, capsid, and nucleocapsid) proteins; the *pol* gene encodes the RNA directed DNA polymerase (reverse transcriptase), and the *env* gene encodes viral envelope glycoproteins. The 5' and 3' LTRs promote transcription and polyadenylation of the virion RNAs, and
10 contains all other cis acting sequences necessary for viral replication. Lentiviruses have additional genes, including *vif*, *vpr*, *tat*, *rev*, *vpu*, *nef*, and *vpx* (in HIV-1, HIV-2, FIV and SIV), which encode accessory proteins.

Adjacent to the 5' LTR are sequences necessary for reverse transcription of the
15 genome (the tRNA primer binding site) and for efficient encapsidation of viral RNA into particles (the Psi (Ψ) site). If the sequences necessary for encapsidation or packaging of retroviral RNA into infectious virions are absent from the viral genome, the result is a cis defect that prevents encapsidation of genomic RNA. However the resulting mutant remains capable of directing synthesis of all virion proteins.

20

The recombinant retroviruses, including lentiviruses, useful in the invention are genetically modified such that structural and infectious genes of the native virus encode nonfunctional gene products. As such, the virus is replication defective, but still contains the encapsidation signal and can be packaged into virions. Following
25 infection of a DC by a recombinant viral vector of the invention, the nucleic acid can integrate into the genome of the host DC. Expressible nucleotide sequences in the integrated viral vector then can be transcribed and translated into polypeptides, which can be expressed, processed, and presented on the surface of the DC.

30 A recombinant retroviral vector, for example, a lentiviral vector, of the invention generally is a "pseudotyped" viral vector containing the envelope of a virus other than the virus contributing the vector genome sequences. The envelope can be derived from any virus, including another retrovirus or lentivirus, and can be amphotropic, xenotropic or ecotropic. Thus, the envelope protein can be an
35 amphotropic env protein such as an MLV env, which allows transduction of cells of a human or other species; or can be an ecotropic env protein, which allows transduction

of mouse or rat cells. The env gene is not contained within the lentiviral vector genome, but is provided by a packaging system used to generate the recombinant vector, for example, by transient cotransfection or by stable, inducible cell lines, to produce a recombinant pseudotyped lentiviral virion, which is useful for transduction of DCs. Packaging cell lines are well known and readily available to those in the art.

Viral envelope proteins useful for pseudotyping a lentiviral vector of the invention include, but are not limited to, Moloney murine leukemia virus (MoMuLV) env, Harvey murine sarcoma virus (HaMuSV) env, murine mammary tumor virus (MMTV) env, gibbon ape leukemia virus (GaLV) env, human immunodeficiency virus (HIV) env, feline immunodeficiency virus (FIV) env, Rous sarcoma virus (RSV) env, and vesicular stomatitis virus env (VSV-G). A pseudotyped lentiviral vector containing an HIV-1 vector genome and a VSV-G envelope is exemplified herein. Additional exemplary lentiviral vectors useful in the methods and compositions disclosed herein are provided in copending U.S. Serial No. 08/936,633, filed September 24, 1997.

If desired, a lentiviral vector can be targeted to a particular site, for example, to a particular cell type, by linking to the env protein an antibody or particular ligand that targets an antigen or receptor expressed by the particular cell type. The viral vector can be made target specific by inserting, for example, a glycolipid or a protein in the env protein. For targeting to dendritic cells, a targeting moiety of particular interest can be specific for CD86 (B7-2), which is expressed at high levels on DCs, but generally is absent on non-APCs. By incorporating a binding domain for CD86 in the coat protein of the viral vector, the virion can be delivered specifically to DCs. CD86 binding domains include its counter-receptors, CTLA-4 and CD28, and antibodies that specifically bind CD86. For example, the nucleotide sequence encoding the binding domain of CTLA-4 can be isolated, for example using restriction endonucleases or PCR amplification, and inserted into an appropriate envelope protein such as VSV-G. Other methods to achieve specific delivery of a viral vector to a target cell will be known to those in the art.

Various cis acting viral sequences are necessary of the viral life cycle, including the Ψ packaging sequence, reverse transcription signals, integration signals, viral promoter, enhancer, and polyadenylation sequences. In addition, a viral vector of the invention contains at least one cloning site, which can be a multiple cloning site

(MCS), so as to facilitate the introduction of a nucleic acid sequence, which can be a heterologous sequence, encoding a polypeptide that is to be expressed in the dendritic cell. The polypeptide can be any polypeptide, particularly a polypeptide antigen or fragment thereof that can be recognized by a cell of the immune system or by an antibody. For example, the polypeptide can be encoded by a "heterologous" nucleic acid sequence, which is a sequence that originates from a species other than that providing the viral vector genome, or can be encoded by a nucleic acid sequence obtained from the same species as virus contributing the vector genome.

10 A nucleic acid sequence introduced into a viral vector of the invention also can encode a marker (reporter) gene product, which can be a selectable marker gene product that can facilitate detection of cells expressing the polypeptide or can be a toxic agent that kills or otherwise inhibits the growth or replication of a cell expressing the polypeptide. As such, a marker gene or gene product can be utilized to
15 assay for the presence of the vector. A selectable marker gene can encode a protein that confers resistance to an antibiotic or other toxic substrate, for example, histidinol, puromycin, hygromycin, neomycin, methotrexate, or the like. A marker also can be a polypeptide that can be assayed by physical means such as by fluorescence or by an enzymatic reaction. Such markers include, for example, β -galactosidase, luciferase,
20 and green fluorescent protein (GFP).

A nucleic acid sequence present in or introduced into a viral vector of the invention also can encode a polypeptide normally expressed by the virus contributing the vector genome, for example, an HIV-1 gag polypeptide expressed from an HIV-1
25 vector of the invention. The viral polypeptide, which can be useful as an antigen, can be the entire viral gene product, or a fragment thereof, or can encode a modified version of the gene product that, for example, is more likely to induce the desired immune response. As such, the encoded polypeptide can be an HIV-1 gag, pol, env, vpr, vif, nef, vpx, tat, rev or vpu gene product, or a corresponding gene product from a related
30 primate immunodeficiency virus, or other retrovirus or lentivirus.

The present invention is based on the discovery that lentiviral vectors can be used to transduce DCs, particularly immature or non-dividing DCs and, therefore, provide vaccines useful for inducing, for example, immunity against HIV. As
35 disclosed herein, the DC based vaccine strategy can induce virus specific cytotoxic T lymphocyte (CTL) immunity. Thus, the invention provides a method of inducing

an immune response in a subject by administering to the subject, a therapeutically effective amount of a DC, or of a progenitor thereof, which has been transduced with a replication defective pseudotyped lentiviral vector having a nucleic acid sequence encoding an antigen that is presented, in whole or in part, on the surface of the dendritic cell. In addition, the invention provides a method of inducing an immune response in a subject by transducing a DC, or a progenitor of a DC, with a pseudotyped lentiviral vector, *in vitro*, including *ex vivo*, or *in vivo*, including *in situ*, to produce a transduced DC, which can be contacted with a T cell to produce an activated T cell. Also provided is a method of activating a T cell by contacting the T cell with a DC transduced with such a pseudotyped lentiviral vector.

The invention also provides a lentiviral vector containing a viral genome encoding nonfunctional env and nef gene products and, if desired, nonfunctional accessory proteins. For example, a pseudotyped HIV-1 vector can comprise and HIV-1 genome having a deletion of the nucleotide sequences encoding the env and nef gene products, or a deletion of nucleotide sequences encoding functional nef, env, vif, vpr and vpu gene products; and a VSV-G env protein. Such vectors can be used for inducing anti-HIV-1 immunity in an individual by contacting DCs of the individual, for example, Langerhans cells, with the vector.

The viral antigen can be a lentiviral antigen, including, but not limited to, a gag, pol, env, vpr, vif, nef, vpx, tat, rev, or vpu gene product, or an immunogenic fragment thereof. A composition or a method of the invention allows the antigen to be presented exclusively to the immune system with multiple, presumably optimal, immunostimulatory signals to amplify many different T-dependent responses, including both proliferative and cytotoxic responses on CD4+ and CD8+ T cells. The nucleic acid of interest can encode a fusion polypeptide consisting of two or more peptides that are linked together, one or both of which can be antigenic when expressed in a DC.

The nucleic acid sequence, which can be a heterologous sequence, also can encode a tumor antigen, which can be specific to the tumor cells. Such tumor antigens can be useful for stimulating an immune response that can be specific for a particular tumor, which can contain malignant cells. A tumor antigen can be encoded by a normal gene, for example, agent that normally is silent depending on the age or stage of development of the individual; a gene encoding a differentiation antigen; a

gene encoding an embryonic or fetal antigen; or a gene encoding a clonal antigen, which normally is expressed only on one or a few types of normal cells. Tumor specific antigens can be encoded by a mutant cellular gene, including an oncogene such as an activated ras oncogene; a tumor suppressor genes such as a mutant p53 gene; or a fusion protein resulting from a deletion or translocation in a chromosome. A tumor specific antigen also can be encoded by a viral genes such as RNA or DNA tumor virus.

In lymphoma, the idiotype of the secreted immunoglobulin serves as a highly specific tumor associated antigen. An "idiotype" is a collection of immunoglobulin variable region determinants that are specific to a particular antibody or limited set of antibodies. The nucleic acid encoding such an antigen can encode a lymphoma specific idiotype. For use with tumor antigens, public policy may indicate that vector based on a lentivirus other than HIV be used in the disclosed methods.

A nucleic acid sequence encoding a polypeptide, for example, an antigen in a vector of the invention is operably linked to the appropriate regulatory elements, such that the sequence is properly transcribed and translated. The term "operably linked" refers to the functional linkage between a regulatory element and the expressible nucleic acid sequence, which encodes the polypeptide. Generally, the expressible nucleic acid sequence is operably linked to a transcriptional promoter, ribosome binding site, polyadenylation signal or other regulatory element as desired, to produce a chimeric gene construct. The expressible nucleic acid sequence also can be under control of either the viral LTR promoter/enhancer signals or of an internal promoter; retained signals within the retroviral LTR allow efficient integration of the vector containing the expressible nucleic acid sequence into the genome of the DC. The promoter sequence or other regulatory element can be homologous or heterologous to the expressible nucleic acid sequence. A wide range of promoters may be utilized, including viral or mammalian promoters. Cell or tissue specific promoters can also be utilized, such as the CD86 promoter. These and other regulatory elements are well known in the art and readily available from commercial sources.

A pseudotyped lentiviral vector of the invention can contain an expressible nucleic acid sequence encoding a cytokine. Cytokines are well known in the art and include a relatively diverse group of soluble proteins and peptides that act as humoral regulators at nanomolar to picomolar concentrations and, under normal or

pathological conditions, modulate the functional activities of individual cells and tissues. Cytokines also can mediate interactions between cells directly, and regulate processes occurring in the extracellular environment. Cytokines can influence the maturation of DCs and are involved in the immune response to an antigen. Thus, a
5 pseudotyped lentiviral vector can contain an expressible nucleic acid sequence encoding a cytokine that regulates the maturation of DCs. Examples of cytokines include, but are not limited to, interleukin-2 (IL-2), IL-3, IL-4, granulocyte macrophage colony stimulating factor (GM-CSF), stem cell factor (SCF), and the Flt-3/Flk-2 ligand (FL). As for any expressible nucleic acid sequence, one encoding a
10 cytokine is operably linked to the appropriate regulatory elements, which can be homologous or heterologous to the nucleic acid sequence encoding the cytokine.

A lentiviral vector of the invention can transfer an expressible nucleic acid sequence encoding a polypeptide of interest, for example, an antigen into a DC such
15 that the polypeptide is expressed by the DC. The expressible nucleic acid sequence can be any nucleic acid molecule, particularly DNA, and can be derived from a variety of sources including genomic DNA, cDNA, a synthetic DNA molecule, RNA, or any combination thereof. Where the nucleic acid sequence is derived from genomic DNA, it can contain one or more introns and, therefore, must be compatible
20 with the processing mechanisms in the DC in which the transcript is produced. Methods for obtaining genomic nucleic acid sequences, cDNA sequence, and RNA, including mRNA, are well known and routine in the art, and include PCR, reverse transcription, and the like. Methods for synthesizing nucleic acid sequences similarly are known and routine.

25 A viral vector of the invention provides a means to transduce a DC or other APC or cell type. As used herein, the term "transduction" refers to the act of introducing a nucleic acid sequence, which can be a viral vector, into a cell, including the genetic change resulting from introduction, incorporation or expression of the
30 introduced (exogenous) nucleic acid sequence in the cell. The exogenous nucleic acid can be present in the cell as an extrachromosomal entity or can be integrated into the genome of the cell. Where the cell is a mammalian cell transduced using a vector of the invention, the genetic change generally is achieved by integration of all or part of the exogenous nucleic acid sequence into the genome of the cell. Such integration
35 can result in stable expression of one or more polypeptides encoded by the nucleic acid sequence, although transient expression also can occur, as can inducible

expression, stage specific expression, or the like, depending, in part, on the regulatory elements operably linked to the expressible nucleic acid sequence. Transduction can be performed *in vivo* or *in vitro*, as disclosed herein.

5 Expression of the nucleic acid of interest occurs as a result of the pseudotyped lentiviral vector entering the DC, and can result in presentation of the encoded polypeptide, or of processed peptide fragments of the polypeptide to class I or class II MHC molecules to form a bimolecular complex recognized by T cells. This complex is transported to and displayed on the surface of the DC. Activation of the DC can be
10 manifested by the expression of adhesion molecules that promote the physical interaction between T cells and dendritic cells; membrane bound growth or differentiation molecules (costimulators) that promote T cell activation; and expression of soluble cytokines such as IL-1 and tumor necrosis factor (TNF). As such, a transduced dendritic cell contains a pseudotyped lentiviral vector containing
15 an expressible nucleic acid sequence, which encodes a polypeptide, for example, an antigen that can be presented to the immune system.

 Contact of a transduced DC of the invention with a T cell results in the production of an activated T cell. As used herein, the term "contacting" means that
20 the DC and the T cell interact under suitable conditions, which can be *in vivo* or *in vitro* conditions, such that T cell activation occurs. For example, the DC can be transduced *in vitro*, including *ex vivo*, then contacted with a T cell *in vivo*. Alternatively, the transduced DC can be contacted with the T cell *in vitro* (see Henderson *et al.*, *supra*; 1996; Reeves *et al.*, *supra*, 1996). Methods for isolating
25 T cells, for example, from an autologous or allogeneic donor, by flow cytometry, panning, antibody magnetic bead conjugates, or the like, are well known, or a T cell line may be employed. The DCs also can be transfected with an expression vector that encodes a protein domain containing addressing information, for example, a ligand for a receptor expressed by activated T cells or a counter-receptor for
30 addressins or selectins, thereby conferring cell type specificity.

 The DC and the T cell are contacted under conditions that allow T cell activation. T cell activation occurs when a polypeptide is presented on an APC such as a DC in the context of MHC class I or class II molecules. A T cell expressing a T
35 cell receptor-CD3 complex undergoes molecular events indicative of T cell activation, including the activation of a src family tyrosine kinase, phosphorylation of

phospholipase C, or the secretion of a cytokines such as IL-2. Culture requirements for T cell activation *in vitro* are well known (Henderson et al., *supra*; 1996; Reeves et al., *supra*, 1996).

5 A method of the invention can include administration of a therapeutically effective amount of a DC or a progenitor thereof transduced with a pseudotyped lentiviral vector containing a nucleic acid sequence encoding an antigen of interest. A method of the invention also can include administration of the pseudotyped lentiviral vector containing a nucleic acid sequence encoding an antigen, or of a T cell activated
10 by a transduced DC. Such a composition can be administered to any subject, particularly a mammalian subject such as a human. As used herein, the term "therapeutically effective amount" means an amount of a composition, which can be a pseudotyped lentiviral vector, a DC cell transduced with such a vector, or a T cell activated by contact with such a vector, sufficient to effect a humoral or cellular
15 immune response against the antigen in the subject. Method of detecting such an immune response in the subject are well known and routine in the art and include, for example, determining antibody titer against the antigen or determining reactivity of immunoeffector cells in the subject.

20 A method of the invention can provide a preventive, therapeutic, or palliative response in the subject. Where the method is performed for preventive purposes, for example, as a vaccination to prevent an infection, methods of determining that a suitable immune response has been generated can be determined as disclosed above or using other methods for identifying that a vaccination has been effective. Where the
25 method is performed for a therapeutic or palliative purpose, due to the subject suffering from a pathological condition, generation of an effective immune response can be identified using the above disclosed methods, or by measuring or observing clinical signs or symptoms associated with a pathology. For example, where the pathology being treated is HIV-1 infection, an effective immune response can be
30 identified by measuring a decrease in the rate of T cell declination in the subject, or an increase in T cell numbers, or by detecting an increase in appetite or, simply, by the subject indicating that he or she feels better.

 The term "immune response" is used herein to refer to a T cell response or a
35 B cell response. The immune response can be detected due to increased serum levels of antibodies to a particular antigen, or to the presence of neutralizing antibodies to

the antigen. The immune response can confer "protective immunity," which is the ability of the serum antibodies and the T cell response induced during immunization to protect, at least partially, against a disease for which the immunization was prescribed. Preferably, the immune response is a cellular response, particularly a
5 cytotoxic T cell (CTL) response.

A method of the invention can be used to stimulate an immune response against a virus in a virally infected subject. A method of the invention can be used to protect against a viral infection by stimulating the immune response against the virus.
10 In addition, a method of the invention can be used to stimulate an immune response against a neoplasm, or can be used stimulate an immune response in a cancer patient so as to minimize the likelihood of tumor metastasis in the subject. As used herein, the term "tumor" means any new growth, including a benign or malignant neoplasm. Benign neoplasms generally are encapsulated and are noninvasive, whereas malignant
15 neoplasms (cancer) seldom are encapsulated, and generally are invasive and can be metastatic. A method of the invention can stimulate an immune response against neoplastic disorders, including, but not limited to, a sarcoma, carcinoma, fibroma, lymphoma, melanoma, neuroblastoma, retinoblastoma, and glioma.

20 A composition useful in performing of the invention, including a pseudotyped lentiviral vector, DCs transduced with such a vector, or T cells activated by contact with such DCs, are administered to a subject. The composition can be administered by any means known to the skilled artisan. In particular, a composition can be administered into the skin of a subject, for example, by intradermal or intraepidermal
25 injection using a fine needle, a trocar or other instrumentation suitable for the particular composition. Where the lentiviral vectors are administered, they can be administered to a patient as packaged virus particles. Alternatively, the lentiviral vector can be administered in the provirus form, particularly integrated in the genome of dendritic cells such as Langerhans cells.

30 The pseudotyped lentiviral vectors generally are replication defective, and are packaged *in vitro*. The packaged virus can be delivered to the subject in order to transduce the dendritic cells of the subject, for example, by intradermal injection. If desired, pseudotyped lentiviral vector comprising a nucleic acid encoding an antigen
35 also can be administered in combination with DCs transduced with the same or another pseudotyped lentiviral vector comprising a nucleic acid encoding an antigen,

or can be administered in combination with T cells activated by DCs transduced with the same or another pseudotyped lentiviral vector comprising a nucleic acid encoding an antigen.

5 The clinical administration of retroviruses has been accomplished by the direct injection of virus into tissue, and by the administration of the retroviral producer cells. Methods for delivering retrovirus and retroviral producer cells to a subject are well known, and include intradermal, intramuscular, intravenous, intraperitoneal, and subcutaneous delivery. The pseudotyped lentivirus comprising a nucleic acid
10 sequence encoding an antigen can be prepared as a formulation containing a pharmacologically effective dose in pharmaceutically acceptable medium, for example normal saline or phosphate buffered saline. The pharmaceutical composition additionally can contain a bactericidal agent, stabilizers, buffers, adjuvants, or the like. In addition, the virus can be administered as a single agent, or in combination
15 with one or more other agents, including a therapeutic or immunostimulatory agent.

 The amount of a viral vector or other composition as disclosed herein that is administered to a subject will vary depending, for example, on the nature of the disease, the frequency of administration, the manner of administration, and the
20 clearance of the agent from the subject. For example, a dose may be administered to a subject weekly or biweekly, or may be fractionated into smaller doses and administered daily or semiweekly to maintain an effective dosage level. Regardless of such factors, however, the composition is administered at a dosage sufficient to induce an immune response in the subject, the particular parameters being determined
25 by phase I and phase II clinical studies as is routine in the art.

 Transduced DCs, which can be xenogeneic, allogeneic, syngeneic or, preferably, autologous, also can be administered to a subject. Dendritic cells can be administered to the site in which stimulation of the immune response is desired, or
30 can localize to the site for treatment following administration to the subject. The dendritic cells can be administered in any physiologically acceptable medium, and generally are administered intradermally, intravascularly, or into a lymph node, which can provide a suitable environment for expansion and differentiation, if desired, and for contacting and activating T cells. Any transplantation or implantation procedure
35 known in the art can be utilized for administering transduced DCs, or T cells activated *in vitro* by contact with such DCs. For example, the selected cells or cells of interest

can be implanted intradermally using a trocar, which contains a wide bore so as to minimize the likelihood of damage to the DCs, or can be surgically implanted into the recipient or subject. If desired, the DCs (or activated T cells) can be administered in an encapsulated form.

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Transplantation or implantation is typically by simple injection through a hypodermic needle (trocar) having a bore diameter sufficient to permit passage of a suspension of cells without damaging the cells or tissue coating. The cells also can be administered, for example, into the peritoneal cavity using a laparoscope, or into any other site such as the thymus, liver, spleen, kidney capsule or a lymph node, depending, for example, on the specific DCs used and desired biological effect. For implantation, the typically the cells are formulated as a pharmaceutical composition, which can include a pharmaceutically-acceptable carrier, containing a sufficient number of cells, usually at least about 1×10^5 cells and, particularly, about 1×10^6 cells or more. The cells can be frozen in liquid nitrogen and stored for long periods of time prior to use, then can be used directly after thawing, or can be expanded, then used.

15

Transduced DCs can be encapsulated prior to transplantation. Although cells generally are microencapsulated, they also can be encased in various types of hollow fibers or in a block of encapsulating material. A variety of microencapsulation methods and compositions are known in the art, including, for example, the use of alginate polymers or agarose to supply the encapsulation compositions. Alginates are linear polymers of mannuronic and guluronic acid residues arranged in blocks of several adjacent guluronic acid residues forming guluronate blocks, and blocks of adjacent mannuronic acid residues forming mannuronate blocks, interspersed with mixed or heterogenous blocks of alternating guluronic and mannuronic acid residues. Generally, monovalent cation alginate salts such as sodium alginate are soluble.

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Divalent cations, such as Ca^{++} , Ba^{++} and Sr^{++} can interact with guluronate, and the cooperative binding of these cations within the guluronate blocks provides the primary intramolecular crosslinking responsible for formation of stable ion-paired alginate gels. Alginate encapsulation methods generally take advantage of the gelling

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- of alginate in the presence of these divalent cation solutions. In particular, these methods involve the suspension of the material to be encapsulated, in a solution of monovalent cation alginate salt, *e.g.*, sodium. Droplets of the solution are then generated in air and collected in a solution of divalent cations, *e.g.*, CaCl_2 . The
- 5 divalent cations interact with the alginate at the phase transition between the droplet and the divalent cation solution resulting in the formation of a stable alginate gel matrix being formed. Generation of alginate droplets has been carried out by a number of methods including, for example, generating droplets by extrusion of alginate through a tube by gravitational flow into a solution of divalent cations.
- 10 Similarly, electrostatic droplet generators, which rely on the generation of an electrostatic differential between the alginate solution and the divalent cation solution, have been used. The electrostatic differential results in the alginate solution being drawn through a tube, into the solution of divalent cations (see, for example, Goosen, Fundamentals of Animal Cell Encapsulation and Immobilization, Chap. 6, pages 114-
- 15 142 (CRC Press, 1993)).

- Further, methods have been described wherein droplets are generated from a stream of the alginate solution using a laminar air flow extrusion device. Specifically, this device comprises a capillary tube within an outer sleeve. Air is driven through
- 20 the outer sleeve and the polymer solution is flow-regulated through the inner tube. The air flow from the outer sleeve breaks up the fluid flowing from the capillary tube into small droplets (see U.S. Patent No. 5,286,495). Viable tissues and cells also have been immobilized in alginate capsules coated with polylysine (*J. Pharm. Sci.* 70:351-354, (1981)) and such coated capsules have been used in pancreatic islet
- 25 transplantation to correct the diabetic state of diabetic animals (*Science* 210:908-909, (1981)).

- As disclosed herein, transduced DCs can be used to activate T cells *in vitro*, then the activated T cells can be introduced into a subject. The adoptive transfer of
- 30 immune cells is well known in the art (Rohane et al., *Diabetes* 44:550-554, 1995). The activated T cells can be administered using methods as described above for delivering DCs, and can be administered in any physiologically acceptable medium. Generally, activated T cells are administered intravascularly, for example,

intravenously, although they can be introduced into a lymph node or other appropriate site such as the site of a neoplasm. Any of the transplantation or implantation procedures as disclosed above or otherwise known in the art can be utilized, and the activated T cells can be administered alone, or in combination with a pseudotyped
5 lentiviral vector containing a nucleic acid sequence encoding an antigen; with a dendritic cell transduced with such a lentiviral vector, or with any other agent such as a therapeutic or immunostimulatory agent.

Gene therapy of DCs offers the promise of new therapies for cancer, AIDS
10 and autoimmune diseases. The use of lentiviral vectors for gene delivery is superior to the use of oncogenic retroviruses because lentiviral vectors enable the transduction of not only dividing but also non-dividing cells, including DCs. As disclosed herein, immature DCs are efficiently transduced (up to 50% at the highest MOI tested) using the HIV-1 dE 3dVx vector, which is pseudotyped by the VSV-G protein and has
15 deletions of the env, nef, vif, vpr, and vpu genes. The efficient transduction of HIV-1 dE 3dVx in non-dividing DCs is due, in part, to replacement of the HIV-1 envelope protein by the VSV-G protein, which eliminates the requirement for Nef for enhanced HIV-infection, to the dispensibility of Vpr for targeting the viral preintegration complex to the nucleus in non-dividing cells because of the functional
20 redundancy of Vpr, matrix protein (MA) and integrase. The requirement of accessory genes for the transduction of growth-arrested or non-dividing cells is not absolute, and differs between individual cell types (Kim et al., *J. Virol.* 72:811-816, 1998; Zufferey et al., *Nature Biotechnol.* 15:871-875, 1997; Gasmi et al., *J. Virol.* 73:1828-1834, 1999).

25 As disclosed herein, transduction did not affect the viability of Mo-DC, and did not significantly alter the immunophenotype of immature Mo-DC or disturb differentiation of immature Mo-DC into mature Mo-DC (see Example 5). As such, the disclosed HIV-1 vector system differs from vaccinia vectors, which can interfere
30 with DC differentiation and cause toxicity. Furthermore, important functional properties of DC, including antigen uptake by immature DC and T cell stimulation by mature DC, were not negatively influenced by transduction with HIV-1 dE 3dVx in Mo-DC.

Cryopreserved immature Mo-DC were transduced by HIV-1 dE 3dVx with an efficiency comparable to that of freshly cultivated immature Mo-DC. In addition, cryopreservation does not adversely effect the isolation/function of DC derived from PBMC, as has been shown recently (Turner et al., *J. Immunol. Meth.* 223:1-15, 1999). Thus, patient samples can be stored for later use, for example, following treatment of a cancer patient with chemotherapy or radiotherapy. Furthermore, the deletion of five genes within the HIV-1 genome greatly improves the safety of the described vector system by eliminating the possibility of generating replication competent HIV-1 during vector production. In comparison, live (replication-competent) attenuated AIDS vaccines, which currently are the most promising vaccine approach regarding their ability to generate a sustained and broadly effective immunity, still present a safety concern due to their uncontrolled replication *in vivo*, leading to AIDS in a significant proportion of vaccinated animals. In contrast, the vector systems disclosed herein are replication-incompetent. Thus, the reachable virus load *in vivo* will be restricted to the vaccinated vector dose, thereby making it possible to control pathogenicity, which is directly linked to viral replication rates.

The following examples are intended to illustrate but not to limit the invention in any manner, shape, or form, either explicitly or implicitly. While they are typical of those that might be used, other procedures, methodologies, or techniques known to those skilled in the art may alternatively be used.

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EXAMPLE 1

EFFICIENT TRANSDUCTION OF CD34+ CELLS WITH A VSV-G PSEUDOTYPED HIV-1 VECTOR

This example describes the methods used for preparing lentiviral vectors and for determining the ability of the vectors to transduce dendritic cells.

Collection of Dendritic Cells

Mobilized peripheral blood was obtained from normal donors with informed consent and Institutional Review Board approval. The procedure for purifying CD34+ cells has been described previously (Lane, T.A., *et al.*, *Blood* 85:275, 1995).

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DCs also were generated from buffy coats of healthy donors by using protocols similar to those previously reported (Bender *et al.*, *J. Immunol. Meth.* 196:121-135, 1996; Romani *et al.*, *J. Immunol. Meth.* 196:137-151, 1996). Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation in FICOLL-PAQUE (Pharmacia; Uppsala, Sweden), and 1×10^8 PBMC were plated per 75 cm^2 tissue culture flask (Costar; Cambridge MA) in RPMI 1640 (Mediatech; Herndon VA) supplemented with 10% heat-inactivated human serum AB (Gemini; Calabasas CA) and 1% nonessential amino acids (Mediatech).

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After 3 hr, the floating cells were removed and the adherent cells were incubated for 7 days with GM-CSF (100 IU/ml; Leukine; Immunex; Seattle WA) and IL-4 (1,000 U/ml; R & D Systems; Minneapolis MN). Cells were fed every 3 days with the same dose of cytokines. At day 5, the nonattached immature DC, being CCR5⁺, CD4⁺, CD86⁺, and HLA-DR⁺ but weak in CD83, were depleted of CD3, CD14 and CD20 cells either by cell sorting (FACStar plus, Becton Dickinson) or by immunomagnetic bead separation (Dynal; Lake Success NY). Negatively selected cells were transferred into 24 well cell culture plates (Costar) and incubated for an additional 2 days in cytokine containing medium. Macrophages were generated from plastic-adherent PBMC after incubation in GM-CSF (100 IU/ml) containing medium for 7 days. Mature DC were generated by incubating immature DC for 1 or 3 days in medium supplemented with LPS (100 ng/ml; Sigma) or TNF- α (100 U/ml; R & D Systems), respectively, in addition to GM-CSF and IL-4 supplementation. For prolonged storage, cells were frozen down in human serum AB supplemented with 10 % (v/v) DMSO using an isopropyl alcohol filled freezing container as recommended by the manufacturer (Nalgene; Rochester NY) and finally placed in liquid nitrogen.

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Mammalian cell lines

Human leukemia T cell line Jurkat, human histiocytic lymphoma cell line U937, human osteosarcoma cell line HOS (ATCC; Manassas VA), HOS.CD4- CCR5 (see Deng *et al.*, *Nature* 381:661-666, 1996), and human leukemic lymphoblast cell

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line CEM-GFP containing a plasmid encoding the green fluorescent protein driven by the HIV-1 long terminal repeat (Gervais et al., *Proc. Natl. Acad. Sci., USA* 94:4653-4658, 1997) were cultivated as recommended by the provider.

5 Construction and Preparation of Lentiviral Vectors

 The construction of the env and nef deleted HIV-1 vector (HIV-1 dE) has been described (Li et al., *J. Hum Virol.* 1:346 (1998)). The env, nef, vif, vpr, vpu deleted HIV-1 vector (HIV-1 dE 3dVx) was constructed as described below.

10 The HIV-1 dE 3dVx vector was derived with the following strategy (all nucleotide numbering is based on the HIV-1 dE sequence, derived originally from the HIV11 NL43 strain) The pBLUESCRIPT plasmid pBS (Stratagene; La Jolla CA) was used as an intermediate plasmid to facilitate downstream subcloning. The Afl III restriction enzyme site present in the pBS plasmid was destroyed by blunting with the
15 large fragment of Klenow polymerase, then the plasmid was reclosed with T4 DNA ligase. The HIV-1 dE vector plasmid (Li et al., *supra*, 1998) was digested with ApaI and XhoI to release the 6.2 kb ApaI/XhoI fragment (nucleotides 2005-8206), which was subcloned into the ApaI/XhoI sites of pBS, which lacked the Afl III site. The Afl III (nucleotide 6054) through the Stu I (nucleotide 6822) region was released from
20 the insert, and the remaining fragment was blunted with Klenow and self-ligated with T4 DNA ligase. This deletion effectively removed the entire *Vpu* ORF (nucleotides 6061-6301) and the 5' end of the *Env* sequence (nucleotides 6220-6822).

 To remove the EcoRI site from the pBS multiple cloning site, so as to utilize
25 the unique EcoRI site present within the insert, the plasmid was digested with SmaI and ClaI, blunted with Klenow, and reclosed with T4 DNA ligase. The resulting plasmid was digested with a combination of Pfl MI (nucleotide 5297) and EcoRI (nucleotide 5741), blunted with Klenow, and religated with T4 DNA ligase. This deletion effectively removed the 3' half of the *Vif* ORF (nucleotides 5297-5621) and
30 the 5' half of the *Vpr* ORF (nucleotides 5561-5741).

The ApaI/XhoI fragment could not be subcloned back into the HIV-1 dE background because of an ApaI site present 3' to the XhoI restriction site. Therefore, the 3' terminal XhoI (nucleotide 8206)/XbaI (nucleotide 9708, MCS) fragment, 1.6 kb in length, was subcloned into the pBS intermediate to generate a contiguous ApaI through XbaI fragment of HIV-1 sequence (lacking the deleted accessory gene sequences). The AgeI fragment (nucleotides 3484-8259), which encompasses the newly deleted accessory gene sequences, then was transferred into the HIV-1 dE background, which had been digested to remove the analogous region flanked by AgeI. The XhoI, 3' ApaI, and 3' AgeI restriction sites are present within an MCS immediately upstream of the GFP ORF that was originally subcloned into the *Nef* slot (Li et al., *supra*, 1998). The resulting construct is the HIV-1 dE 3dVx vector.

Vesicular stomatitis virus G protein (VSV-G) pseudotyped HIV-1 vectors were prepared by calcium phosphate based cotransfection of 293T cells (5×10^6 cells; 75 cm² tissue culture flask, Costar) with plasmids expressing VSV-G and the modified HIV-1 genome under investigation containing the (enhanced) green fluorescent protein (EGFP) gene from *Aequorea victoria*. Cell culture supernatants were collected at 72 hr posttransfection. The titers were determined on transduced CRFK cells (ATCC) as measured by GFP expression.

In other experiments, VSV-G pseudotyped HIV-1 vectors were prepared by cotransfecting COS cells by electroporation with plasmids expressing VSV-G and an env defective HIV-1 genome expressing the GFP gene (FIG. 1). A similar MLV-based, VSV-G pseudotyped retroviral vector was prepared (FIG. 1). Cell culture supernatants were collected at 72 hr posttransfection and titered on Hela cells by assaying for GFP expression.

Cell Transduction

In some experiments, CD34+ cells (10^6 /ml) were transduced with HIV-1 and MLV vectors at MOI's of 0.5 to 1 in the presence of recombinant human cytokines (GM-CSF 10 ng/ml; SCF 40 ng/ml; and IL-3 10 ng/ml) and 4 µg/ml protamine sulfate). The cells were transduced for 1 to 2 hr at 26 to 28°C while centrifuging at

2400xg, washed 5 times with IMDM containing 10% FCS after 24 hr. Cells were cultured for another 24 hr before FACs analysis and methylcellulose colony assays.

- In other experiments, monocyte-derived dendritic cells were transduced with
5 HIV-1 vectors at multiplicities of infection (MOI) of 5, if not stated otherwise, in the presence of 4 µg/ml polybrene (Sigma, Saint Louis, MO), GM-CSF and IL-4 for 16 hr at 37 °C. DC were washed and incubated for an additional 4 days in medium supplemented with GM-CSF and IL-4.

10 Assays

- DNA was extracted from CD34+ population after transduction. DNA was amplified using GFP specific primers in conditions recommended by the manufacturer. The PCR was done by 94°C for 2' followed by 30 cycles of 94°C for 30 min, 56°C for 30 min, and 72°C for 1 min. DNA products were analysis on
15 1% agarose gel and visualized by UV.

Infection efficiency

- The infection efficiencies of HIV-1 and MLV vectors pseudotyped with VSV-G proteins of CD34+ cells obtained from mobilized peripheral blood of normal
20 donors was compared. The multiplicity of infection was kept the same by using the same titer obtained by GFP expression in Hela cells. HIV-1 vector packaged in VSV-G showed a five- to tenfold greater transduction of CD34+ cells compared with that for the VSV-G pseudotyped MLV retroviral vectors as measured by DNA PCR. Furthermore, the HIV-1 vector induced threefold higher expression level of GFP as
25 indicated by FACs analysis of the transduced cells.

Immunofluorescence

- Immunophenotyping of cells was accomplished by using phycoerythrin (PE) conjugated, anti-CCR5 (2D7), anti-CD4 (RPA-T4), anti-CD80 (L307.4), anti-CD86
30 (FUN-1), anti-HLA-DR (L243), isotype control antibody (Becton Dickinson; Mountain View CA), anti-CD40 (EA-5; Biosource Intl.; Camarillo CA) and anti-CD83 (HB 15A; Immunotech; Marseille, France). The analyses were carried out on a flow cytometer (Epics Elite, Coulter).

Viability assays

The number of viable cells was determined by trypan blue exclusion assay (Sigma). Cells were observed under a Nikon UFX-IIA microscope (Tokyo, Japan). Apoptotic cells were stained with annexin V-PE (PharMingen; San Diego CA) and
5 quantitated by FACS.

Phagocytosis of latex beads

Immature DCs at a concentration of 10^5 cells, with or without prior transduction, were coincubated with 5×10^6 red fluorescent microspheres (diameter
10 $1 \mu\text{m}$, 2.5% solid, carboxylate-modified latex, Sigma) for varying periods of time. Alternatively, the cells were exposed to 1.0 % (w/v) sodium azide and red fluorescent microspheres to distinguish nonspecifically bound beads from phagocytosed beads. At the end of the assay, cells were separated from unengulfed beads by density gradient centrifugation (Steinkamp et al., *Science* **115**: 64-66, 1982) and analyzed by
15 FACS.

Mixed leukocyte reaction

To assess the antigen-presenting cell function of DC, irradiated mature DC (2000 rad, cesium 137 source) at varying concentrations were coincubated with
20 allogeneic peripheral blood leukocytes (PBL; 1×10^5) in 96-well flat or U-bottom tissue culture microplates (Costar) for 5 days. ^3H -thymidine ($1 \mu\text{Ci/well}$; DuPont NEN, Boston, MA) was added 18 hr before harvest, using an automated cell harvester (Skatron, Sterling, VA). Incorporation of ^3H -thymidine into the cells was quantified using a β -counter (Beckman; Fullerton CA).

25

Statistics

Statistical significance was determined by using Student's t-test. All comparisons were two-tailed, and a P value of less than 0.05 was considered significant.

EXAMPLE 2
GENERATION OF MULTILINEAGE PROGENY CELLS
FROM TRANSDUCE PROGENITOR CELLS

5

To determine if multipotential hematopoietic progenitor cells were transduced by the HIV-1 dE-GFP vector, and to ascertain the fate of GFP expression in lineage committed cells, colonies of granulocytes/macrophages (CFU-GM) and colonies of erythrocytes (CFU-e) derived from the sorted CD34+ cells expressing GFP were assayed by immunomicroscopy.

10

CD34+ cells expressing GFP were plated at 2×10^5 cells/ml in IMDM supplemented with 10% FCS, penicillin/streptomycin (100 nits/ml). The cells were cultured in the methylcellulose plates (Stem Cell Technology) in the presence of combination of the cytokines mentioned above plus IL-6 (50 ng/ml) and Epo 2-3 units/ml for myeloid cell differentiation. For differentiation of DCs, TNF- α (100 units/ml), GM-CSF (10 ng/ml, SCF (40 ng/ml), and IL-4 (400 units/ml) were added to the medium. These cytokines were added to the cultures every 48 hr and the cells expanded as necessary for the growth of DCs.

15

20

For fluorescence microscopy, the cells growing at day 14 in the presence of cytokines for DCs differentiation were stained as above using either antibody CD1a-PE, or antibody CD14a-PE. After staining, the cells were washed and resuspended at 10^5 cells/ml. Approximately 10^4 cells were applied to standard glass microscope slides, and observed using a Nikon FXA photomicroscope. Colonies were collected at day 14 postinfection to detect GFP expression. The results demonstrated that about 40 to 60% of cells in each type of colony assayed expressed GFP, indicating that the progenitor cells were stably transduced and maintained high levels of gene expression from the HIV-1 LTR.

25

30

To test whether the expression of HIV viral proteins could interfere with the differentiation capacity of CD34+, transduced CD34+ cells were sorted according to the GFP expression. GFP+CD34+ cells were plated on methylcellulose plates. In comparison with nontransduced CD34+ cells, the numbers of colonies for CFU-GM and CFU-e were decreased about 10 to 20% (Table 1). Immunofluorescence

35

microscopy showed that high levels of HIV-1 expression led to apoptosis of the progeny cells, which may account for 10 to 20% loss of the cells.

TABLE 1

5 **The Effect of HIV-1 Transduction on Colony Formation of CD34+ Cells**

	<u>Vectors</u>	<u>CFU-GM</u>	<u>BFU-e</u>
	LNL	135	112
	MLV (control)	156	125
10	HIV-1	128	104
	HIV-1	121	102
	HIV-1	108	89
	HIV-1	105	92

15 Several different cytokine combinations have been reported to induce differentiation of DCs from precursor CD34+ cells in peripheral blood. Cytokine combinations which gave about 50 to 60% DCs were chosen (Henderson, *Cancer Res.* 56:3763, 1996). This combination allowed the maximal proliferation of DCs while retaining the CD1a^{bright}CD14- phenotype. To generate DCs that express GFP,

20 CD34+ cells were purified from mobilized peripheral blood and transduced with HIV-1 dE vector. GFP expressing CD34+ were collected by cell sorting and used to differentiate into DCs in the presence of combination cytokines. DCs expressing GFP were identified by cell morphology such as cytoplasmic tails and by negative CD14 staining. About 50 to 70% of the culture repeatedly displayed typical DC morphology and expressing GFP during the 6 week culture period. Since fluorescence detection of

25 GFP requires high levels of gene expression, DCs have sufficient transcriptional factors to ensure high level expression of genes from the HIV-1 LTR promoter. The high level of HIV-1 gene expression did not interfere with DC proliferation, in contrast to conclusion from a previous publication (Granelli-Piperno *et al.*, *Proc. Natl. Acad. Sci. USA* 92:10944, 1995).

30

VSV-G pseudotyped HIV-1 vectors are efficient in transducing CD34+ cells with or without cytokine stimulation. In the studies described here, the feasibility of stable gene transfer into human DCs by HIV-1 vectors was demonstrated. The

35 process of HIV transduction and expression of HIV genes does not alter or influence the generation or differentiation of DCs from CD34+ cells.

EXAMPLE 3

MO-DC FROM LEUKAPHERESIS SAMPLES

- 5 DCs were generated from monocytes in mobilized PBMC of healthy donors and breast cancer patients. From 2×10^{10} PBMC collected by leukapheresis, 10^9 Mo-DC were obtained. Mononuclear cells were twice purified by FICOLL-HYPAQUE gradient centrifugation and monocytes were isolated by adherence to plastic in RPMI alone overnight. Nonadherent cells were removed by vigorous washes and cultured for 7 days in RPMI with 10% fetal calf or human AB serum containing 100 ng/ml each of GM-CSF and IL-4. Within 3 days, the cells detached from the plastic and became a suspension culture. However, if the cells were replated onto a fresh tissue culture flask or glass, they reattached and became characteristically dendritic. Detailed analysis of over 15 Mo-DC preparations from 10 healthy donors and 5 breast cancer patients revealed no significant difference in surface CD phenotypes (Table 2).

TABLE 2
CD Phenotypes of Mo-DC are Characteristic of Mature DC

20	MHC antigens	HLA Class I++ and Class II++
	Costimulatory molecules	CD80+ and CD86++
	T cell markers	CD2-, CD3-, CD4-, CD8-, CD95+
	B cell markers	CD19- and CD83
25	NK markers	CD16-, CD 56- and CD57-
	Myeloid markers	CD14-, CD13+, CD64-
	ukocyte markers	CD45RA-, CD45RO+, CD32+

In vitro Cytokine Requirements for Maturation of DC

- 30 Antigen presenting function of Mo-DC was enhanced by adding IL-2, IL-3, SCF and FL to culture medium containing GM-CSF plus IL-4. Consistent with the prevailing idea that GM-CSF is not the major growth factor for DC *in vivo*, GM-CSF could be replaced by FL and SCF in the differentiation of monocytes to DC, although IL-4 must be present. There was no difference between the surface CD phenotypes of Mo-DC derived from FL and SCF from GM-CSF, and they were all equally effective APC.

Low Density Mature DC Found Only in Mobilized PBMC

Putative novel DC precursors in 5 mobilized PBMC have been identified. A population of small (similar to a medium sized lymphocyte), round, low density cells representing up to 5 to 10% of the total cell number was isolated over metrizimide gradients (Bender *et al.*, *J. Immunol. Meth.* 196:121, 1996). The cells were devoid of lineage markers for T cells, B cells and NK cells (CD3-, CD19-, CD16/56-) but were negative for DC14^{dim} and stained strongly for HLA-DR. Two samples were CD40-CD80-CD86-. As with monocytes, the fresh cells failed to elicit a MLR. After a 7 day culture in GM-CSF and IL-4, however, they became CD14-, CD40+, CD80+, CD86+ and HLA-DR+DC. These cultured low density DC were distinct from Mo-DC derived from the same donor. They were smaller and had dense nuclei. Furthermore, when cultured in an additional third cytokine such as IL-2 and IL-3, they underwent dramatic morphological changes while Mo-DC were not similarly affected. These data indicate that the low density cells may be a less differentiated precursor of DC than monocytes in the differentiation pathway of the myeloid DC.

The low density DC14+ HLA-DR+ cells in the three samples constitutively expressed CD40, CD80 and CD86. The fresh cells were fully competent APC, inducing a MLR without further manipulation or exposure to cytokines. These, too, upon culture with GM-CSF and IL-4 for 7 days became dendritic. The low density DC represented 5 to 10% of the cells in mobilized PBMC, a yield that is unprecedented for other known DC precursors. Thus, mobilized blood is an enriched and invaluable source for DC and their precursors.

25

EXAMPLE 4

EFFICIENT TRANSDUCTION AND TRANSGENE EXPRESSION OF MATURE MO-DC WITH PSEUDOTYPED HIV VECTORS

A preparation of Mo-DC, verified to be >95% homogeneous, was transduced with the HIV-1 dE vector in the presence of GM-CSF (100 ng/ml) and IL-4 (100 ng/ml) and 4 mg/ml polybrene. The cells were transduced at a MOI of three times for 30 min. at 25°C while centrifuging at 2400xg. Cells were washed five times with medium and cultured in RPMI 1640 containing 10% human AB serum, GM-CSF and IL-4. Mo-DC were fixed with 4% paraformaldehyde in PBS at 2, 3, 4, 5 or 6 days after transduction. The percent of GFP expressing Mo-DC among unselected

35

Mo-DC was determined by counting under fluorescent microscopy. Greater than 40% of the cells expressed GFP at 4 days post-transduction.

TABLE 3

5 **Transduction of Mature Mo-DC With the VSV(G)-pseudotyped HIV-1 Vector**

	Days After Transduction	% GFP Positivity
10	2	11.6
	3	33.6
	4	43.7
	5	33.7
	6	31.0

15 In summary, HIV antigens have been stably introduced into human DC by HIV-1 dE vectors pseudotyped with the VSV-G protein, which allows highly efficient transduction into the CD34+ progenitor cells as well as Mo-DC. The results demonstrate that an HIV-1 dE vector encoding HIV-1 antigens and a GFP reporter gene successfully transduces CD34+ cells and Mo-DC with high efficiency relative to
20 murine retroviral vectors; and that HIV-1 vector transduction does not interfere with CD34+ cells differentiation *in vitro* nor alters the morphology or surface CD phenotype of Mo-DC. Four preparations of DC from CD34+ precursors are able to support high-level, stable expression of genes driven by the HIV-I LTR, indicating that sufficient Sp1 or compensatory transcriptional factors are present in these cells.
25 The transduced genes are likely to be integrated since they are also expressed in other subsets of progeny cells, such as macrophages and erythrocytes.

EXAMPLE 5

30 **HIV-1 dE 3dVx EFFICIENTLY TRANSDUCES DIVIDING AND NON-DIVIDING CELLS**

Transduction efficiency of VSV-G pseudotyped HIV-1 vector dE 3dVx in dividing and non-dividing cells

35 The VSV-G pseudotyped HIV-1 dE 3dVx vector, which has deletion of the env, nef, vif, vpr and vpu genes was utilized for these studies. The additional

deletions as compared to HIV-1 dE further reduce the possibility of generating pathogenic HIV-1 recombinants during the production of HIV-derived vectors.

Transient transfection of 293T cells resulted in dE 3dVx titers of 1×10^7 to 4×10^7 transducing units/ml on CRFK cells. Transduction of dE 3dVx at a MOI of 5 in different cell lines resulted in 30% to 90% transduction efficiency. The transduction efficiency of HIV-1 dE 3dVx in the CD4⁻ and CCR5⁻ HOS cell line was comparable to the Jurkat cells and the CD4⁺ and CCR5⁺ HOS.CD4-CCR5 cell line, but was markedly higher when compared to the U937 and CEM cell line. These results indicate that, due to VSV-G pseudotyping, transduction was independent of CD4 or HIV-1 relevant chemokine receptors.

In addition to transduction of dividing cells, as demonstrated above, HIV-1 dE 3dVx transduced non-dividing Mo DC and Mo macrophages. Mo DC resembled immature DC, as determined by their morphology, immunophenotype, and functional property. The transduction efficiency of HIV-1 dE 3dVx was comparable in Mo DC and in Mo macrophages, and was over 20% in the respective cell population (Table 4). By increasing the MOI to 50, approximately 50% of Mo DC were transduced by HIV-1 dE 3dVx (Table 4). Storage of Mo DC in liquid nitrogen prior to transduction did not affect transduction efficiency. The transduction efficiency of dE 3dVx in mature Mo DC was more than four-fold lower when compared to the transduction efficiency in immature Mo DC (Table 4).

TABLE 4

Transduction efficiency of HIV-1 dE 3dVx in Mo DC and Mo macrophages

	Mo macrophages	Mo DC	
		mature	immature
MOI 5	22.5 ± 4	.5 ± 9	5.4 ± 3
MOI 50	n.d.	.5 ± 4	n.d.

5 n.d.: not determined. DCs were transduced either before (immature Mo DC) or after differentiation (mature Mo DC).

Viability of DC after Transduction with HIV-1 dE 3dVx

10 Since some but not all HIV-1 strains may exert a cytopathic effect on cell populations, including DC (Canque et al., *Blood* 88:4215-4228, 1996; Blauvelt et al., *Clin. Invest.* 100:2043-2053, 1997), it was further investigated whether HIV-1 dE 3dVx transduction in immature Mo DC may affect cell viability. The percentage of necrotic as well as apoptotic Mo DC was not modified by transduction with HIV-1 dE 3dVx as determined by trypan blue exclusion assay and annexin V
 15 staining, respectively (Table 5). Additionally, even at a higher MOI of 50, at which 50% of immature Mo-DC were transduced, no significant effect on cell viability could be detected.

TABLE 5
Viability of DC after transduction with HIV-1 dE 3dVx

5

Cell labeling (%)		
DC transduction	Trypan Blue	Annexin V
mock	6.4 ± 5	16.6 ± 8
dE 3dVx	6.0 ± 7	16.0 ± 6

Mean and standard deviation of at least two experiments are shown.

Immunophenotype and Differentiation of HIV-1 dE 3dVx Transduced Immature DC

GM-CSF plus IL-4 cultured monocytes showed moderate to high expression
 10 of HLA-DR, CD86 and CD40 but only little to no expression of CD83 and CD80,
 thus resembling immature or not fully mature Mo DC (Picki et al., *J. Immunol.*
 1573850-3859, 1996). Transduction of immature Mo-DC with HIV-1 dE 3dVx did
 not significantly alter their immunophenotype. Furthermore, the differentiation of
 immature Mo-DC into mature Mo-DC, which is accompanied by an increase in
 15 HLA-DR, CD86, CD83 and CD80 expression, was not affected by transduction with
 HIV-1 dE 3dVx.

Functional Properties of dE 3dVx Transduced DC

Large particle uptake, a characteristic functional property of immature DC,
 20 was not altered by HIV-1 dE 3dVx transduction. The results demonstrate that, after
 an incubation period of 2 hr, a plateau was reached, at which about 60% of the
 Mo-DC population had phagocytosed red fluorescent latex beads, regardless whether
 they were transduced with HIV-1 dE 3dVx.

25 In the allogeneic mixed leukocyte reaction (MLR) mature Mo-DC were
 approximately two-fold more potent in stimulating T-cell proliferation than immature

Mo-DC, while Mo macrophages showed no significant T-cell stimulation. In addition, transduction prior to maturation of immature Mo-DC did not affect their ability to stimulate T cell proliferation in the allogeneic MLR when compared to untransduced Mo-DC, under the assay conditions used.

5

These results demonstrate that accessory genes are dispensable for the efficient transduction of VSV-G pseudotyped HIV-1 based vectors in Mo-DC and, that vector transduction does not affect phenotype or function of these cell type.

10

EXAMPLE 6

FULLY FUNCTIONAL IMMATURE DC CULTURED FROM BLOOD MONOCYTES (MO-DC) WILL GENERATE HIV-SPECIFIC CD8+ CTL

To generate fully functional immature DC, adherent monocytes are in
15 GM-CSF and IL-4 for 5d before infection with the HIV-1 vector uniquely capable of integrating into noncycling cells. Previous experiments have demonstrated that Mo-DC efficiently expressed the GFP reporter gene driven from the HIV LTR. The Mo-DC are routinely >95% homogeneous and up to 10^9 cells can be prepared from each leukapheresis sample. CD8+ T cells are positively selected using
20 immunomagnetic beads. T cells are isolated with a CD8 peptide₅₉₋₇₀- specific monoclonal antibody and eluted from the magnetic beads with the corresponding peptide. Nonspecific activation with this procedure has been noted and the purified T cells were more homogeneous than preparations isolated by negative selection. CD8+ T cells are admixed with virus-transduced DC (v-DC) at a ratio of 10:1 and incubated
25 for 4 days. Selective expansion of virus-specific T cells is performed in a low dose of IL-2 and IL-7 with weekly restimulation with v-DC plus cytokines for up to 7 weeks. Virus-specific cytotoxicity is determined by a standard chromium release assay, using virus-infected HLA A2.1-expressing Jurkat cells (A2.1-Jurkat) as positive targets and for negative control targets, the uninfected A2.1 Jurkat as well as A2.1 melanoma
30 cell. To determine whether the CTL response is broadly specific, the ability of the T cells to lyse A2.1-Jurkat pulsed with known A2.1-restricted epitopes of *gag* and *pol* is tested (e.g., *pol*: p476-484; p652-660; *gag*, p77-85). In the event that the virus-specific CTL reactivity was not directed to known epitopes, the novel epitopes are identified by pulsing A2.1-Jurkat cells with a panel of nanomeric peptides
35 overlapping by three residues encoding known immunogenic regions of *gag*, *pol* or

accessory proteins (e.g., rev, tat, vif). These stably transduced Mo-DC can be used for repeated immunization *in vivo* or for *ex vivo* priming of CTL for adoptive T cell therapy.

5

EXAMPLE 7**POPULATIONS OF FRESH (UNCULTURED) DC AND
OTHER COMMITTED DC PRECURSORS ARE EFFECTIVE
ANTIGEN PRESENTING CELLS AFTER VIRUS TRANSDUCTION**

10 Peripheral blood mononuclear cells (PBMC) are obtained from volunteers given G-CSF. Fresh DC populations are isolated by density gradient centrifugation followed by immunodepletion of nonmyeloid lineage cells. Both fresh mature and immature DC, distinguished by their ability to present alloantigens in a mixed lymphocyte reaction (MLR) and expression of accessory molecules, are transduced
15 with the lentiviral vectors. The ability of untransduced immature DC to differentiate into immunocompetent DC by MLR and expression of appropriate costimulatory and accessory molecules is then determined. If necessary, cytokines will be incorporated into the viral vectors such as flt3 ligand (FL) or IL-4 that has been shown to induce maturation of these DC precursors. Ultimately, all transduced DC are tested for their
20 ability to generate HIV-specific CTL.

EXAMPLE 8**IN VIVO TRANSDUCTION IN A MOUSE MODEL**

25 Mice are immunized with syngeneic DC transduced with the HIV vector containing the CMV promoter and virus-specific CTL activity is measured in the spleen and lymph node. For proof of concept of *in vivo* transduction, mice are immunized *in vivo* with the vector, after daily injections of FL designed to increase the number of DC precursors *in vivo*. Treatment of mice with Flt3 ligand (Flt3L)
30 greatly increased the numbers of different subpopulations of functionally mature dendritic cells (Maraskovsky *et al.*, *J. Exp. Med.* 184:1953, 1997). Immune responses in mice generated by *in vivo* transduction of with or without Flt3L treatment with HIV vectors expressing Env antigens are compared.

35 Eight week old female Balb/c mice are injected subcutaneously once daily with either mouse serum albumin (MSA) (1 µg) or with MSA plus 10 µg of flt3L for

- nine consecutive days. At days 0 and 7, mice are injected with VSV-G/HIV-1 vectors (10e8 TCID₅₀/animal). On day 17, blood is collected to test for env binding and virus neutralizing antibodies, and splenocytes are isolated to test for CTL activity or CD4 helper activity. DCs are also expanded from the bone marrow and tested for APC function. For CTL assays, BALB/c.3T3 fibroblasts are transduced with the VSV/HIV-1 vector to be used as target cells. For CD4 T-helper activity, the splenocytes are restimulated *in vitro* with autologous, irradiated, vector transduced DC for three days, and assayed for proliferation by ³H-thymidine incorporation and cytokine production using cytokine ELISAs (IL-2 or γ -IFN for T_H1 response, IL4 for T_H2 response). ELISA antibodies to MN gp120 and neutralizing antibodies against laboratory strains MN, IIIB, SF2) and primary isolates are measured. To determine whether the T helper response is Type I or II, cytokine production by splenocytes is determined by intracellular staining with cytokine-specific mAb after treating for 4 hours with PMA (20 ng/ml) plus ionomycin (1 μ m) in the presence of monensin. Cells are then fixed, permeabilized, and stained with Cytochrome anti-mouse CD4, FITC anti-mouse γ -IFN and PE anti-mouse IL-4 for analysis by flow cytometry.

- To mobilize DC with FL, mice are injected subcutaneously once daily with 10 μ g of FL for nine consecutive days. On days 0 and 7, mice are injected with VSV(G)/HIV-1 vectors (10e8 TCID₅₀/animal). On day 17, blood will be collected to test for Env binding and virus neutralizing antibodies, and splenocytes are isolated to test for CTL and CD4 T helper cell activity.

- It will be apparent to those skilled in the art that various modifications and variations can be made to the compositions and processes of this invention. Thus, it is intended that the present invention cover such modifications and variations, provided they come within the scope of the appended claims and their equivalents.

What is claimed is

1. A method of inducing an immune response against an antigen in a subject, comprising administering to the subject, a therapeutically effective amount of a dendritic cell, or of a progenitor thereof, which is transduced with a replication defective pseudotyped lentiviral vector,

said vector comprising a nucleic acid sequence encoding an antigen, which is expressed in said dendritic cell and is presented on the surface of the dendritic cell, whereby an immune response is induced against the antigen in the subject.

2. The method of claim 1, wherein the dendritic cell is an immature dendritic cell.

3. The method of claim 1, wherein the dendritic cell is a Langerhans cell.

4. The method of claim 1, wherein the dendritic cell is a non-dividing dendritic cell.

5. The method of claim 1, wherein the progenitor of a dendritic cell is a CD34⁺ cell.

6. The method of claim 1, wherein the pseudotyped lentiviral vector comprises a nucleic acid encoding a cytokine.

7. The method of claim 6, wherein the cytokine is selected from the group consisting of interleukin-2, interleukin-3, interleukin-4, Flt-3/Flk-2 ligand, granulocyte macrophage colony stimulating factor, and stem cell factor.

8. The method of claim 1, wherein the antigen is a tumor antigen.

9. The method of claim 1, wherein the antigen is a fusion polypeptide comprising more than one antigen.

10. The method of claim 1, wherein the antigen is a lentiviral antigen or a fragment thereof.

11. The method of claim 10, wherein the lentiviral antigen is a human immunodeficiency virus-1 (HIV-1) antigen.

12. The method of claim 10, wherein the lentiviral antigen is selected from the group consisting of a gag, pol, env, vpr, vif, nef, vpx, tat, rev, vpu gene product and a fragment thereof.

13. The method of claim 1, wherein the pseudotyped lentiviral vector contains an envelope protein selected from the group consisting of a vesicular stomatitis virus-G (VSV-G) protein and a Moloney leukemia virus (MLV) protein.

14. The method of claim 1, wherein the pseudotyped lentiviral vector is an HIV-1 vector.

15. The method of claim 14, wherein the pseudotyped HIV-1 vector comprises a VSV-G env protein.

16. The method of claim 14, wherein the pseudotyped HIV-1 vector comprises an HIV-1 genome encoding nonfunctional HIV-1 env and nef gene products.

17. The method of claim 14, wherein the pseudotyped HIV-1 vector comprises an HIV-1 genome encoding nonfunctional HIV-1 env, nef and at least three accessory gene products.

18. The method of claim 17, wherein the pseudotyped HIV-1 vector comprises an HIV-1 genome encoding nonfunctional HIV-1 env, nef, vif, vpr and vpu gene products.

19. The method of claim 18, said pseudotyped HIV-1 vector comprising a VSV-G envelope protein.

20. The method of claim 14, wherein the pseudotyped HIV-1 vector contains a nucleotide sequence heterologous to HIV-1.

21. The method of claim 1, wherein the pseudotyped lentiviral vector is a non-HIV lentiviral vector.

22. A method of inducing an immune response against an antigen in a subject, comprising:

transducing a dendritic cell, or a progenitor of a progenitor thereof, with a pseudotyped lentiviral vector,

said vector comprising a nucleic acid sequence encoding an antigen, which is expressed in said dendritic cell and is presented on the surface of the dendritic cell

to produce a transduced dendritic cell; and

contacting the transduced dendritic cells with a T cell to produce an activated T cell,

wherein at least one of the pseudotyped lentiviral vector; the transduced dendritic cell, or progenitor thereof; and the T cell, are administered to the subject.

23. The method of claim 22, wherein the contacting occurs *in vivo*.

24. The method of claim 22, wherein the pseudotyped lentiviral vector or the transduced dendritic cell, or progenitor thereof, is administered to the subject intradermally.

25. The method of claim 22, wherein the transducing occurs *in vivo*.

26. The method of claim 22, wherein the pseudotyped lentiviral vector is administered to the subject intradermally.

27. The method of claim 22, wherein the transducing occurs *in vitro*.

28. The method of claim 22, wherein the contacting occurs *in vitro*.

29. The method of claim 22, wherein the dendritic cell is an immature dendritic cell.

30. The method of claim 22, wherein the dendritic cell is a Langerhans cell.

31. The method of claim 22, wherein the dendritic cell is nondividing dendritic cell.

32. The method of claim 22, wherein the progenitor of a dendritic cell is a CD34⁺ cell.

33. The method of claim 22, wherein the pseudotyped lentiviral vector comprises a nucleic acid encoding a cytokine.

34. The method of claim 33, wherein the cytokine is selected from group consisting of interleukin-2, interleukin-3, interleukin-4, Flt-3/Flk-2 ligand, granulocyte macrophage colony stimulating factor, and stem cell factor.

35. The method of claim 22, wherein the antigen is a tumor antigen.

36. The method of claim 22, wherein the antigen is a fusion polypeptide comprising more than one antigen.

37. The method of claim 22, wherein the antigen is a lentiviral antigen or a fragment thereof.

38. The method of claim 37, wherein the lentiviral antigen is a human immunodeficiency virus-1 (HIV-1) antigen.

39. The method of claim 37, wherein the lentiviral antigen is selected from the group consisting of a gag, pol, env, vpr, vif, nef, vpx, tat, rev, vpu gene product and a fragment thereof.

40. The method of claim 22, wherein the pseudotyped lentiviral vector contains an envelope protein selected from the group consisting of a vesicular stomatitis virus G (VSV-G) protein and a Moloney leukemia virus (MLV) protein.

41. The method of claim 22, wherein the pseudotyped lentiviral vector is an HIV-1 vector.

42. The method of claim 41, wherein the pseudotyped HIV-1 vector comprises a VSV-G env protein.

43. The method of claim 41, wherein the pseudotyped HIV-1 vector comprises an HIV-1 genome encoding nonfunctional HIV-1 env and nef gene products.

44. The method of claim 41, wherein the pseudotyped HIV-1 vector comprises an HIV-1 genome encoding nonfunctional HIV-1 env, nef and at least three accessory gene products.

45. The method of claim 44, wherein the pseudotyped HIV-1 vector comprises an HIV-1 genome encoding nonfunctional HIV-1 env, nef, vif, vpr and vpu gene products.

46. The method of claim 45, said pseudotyped HIV-1 vector comprising a VSV-G envelope protein.

47. The method of claim 41, wherein the pseudotyped HIV-1 vector contains a nucleotide sequence heterologous to HIV-1.

48. The method of claim 22, wherein the pseudotyped lentiviral vector is a non-HIV lentiviral vector.

49. A method of activating a T cell, comprising contacting a T cell with a dendritic cell, or a progenitor of a dendritic cell,
said dendritic cell, or progenitor thereof, having an antigen on its surface,
wherein the dendritic cell, or progenitor thereof, contains a pseudotyped lentiviral vector comprising a nucleic acid sequence encoding the antigen,
whereby contacting the T cell with the dendritic cell, or progenitor thereof, results in activating the T cell.

50. The method of claim 49, wherein the dendritic cell is an immature dendritic cell.

51. The method of claim 49, wherein the dendritic cell is a Langerhans cell.

52. The method of claim 49, wherein the dendritic cell is a non-dividing dendritic cell.

53. The method of claim 49, wherein the progenitor of a dendritic cell is a CD34⁺ cell.

54. The method of claim 49, wherein the activating occurs in a subject *in vivo*.

55. The method of claim 54, wherein the dendritic cell, or progenitor thereof, is administered to the subject.

56. The method of claim 55, wherein the dendritic cell is administered intradermally.

57. The method of claim 54, wherein the dendritic cell, or progenitor thereof, is transduced *in situ* with the vector.

58. The method of claim 57, wherein the dendritic cell, or progenitor thereof, is a Langerhans cell in the skin of the subject.

59. The method of claim 49, wherein the activating occurs *in vitro*.

60. The method of claim 49, wherein the pseudotyped lentiviral vector comprises a nucleic acid encoding a cytokine.

61. The method of claim 49, wherein the antigen is a tumor antigen.

62. The method of claim 49, wherein the antigen is a fusion polypeptide comprising more than one antigen.

63. The method of claim 49, wherein the antigen is a lentiviral antigen or a fragment thereof.

64. The method of claim 63, wherein the lentiviral antigen is a human immunodeficiency virus (HIV-1) antigen.

65. The method of claim 63, wherein the lentiviral antigen is selected from the group consisting of a gag, pol, env, vpr, vif, nef, vpx, tat, rev, vpu gene product and a fragment thereof.

66. The method of claim 49, wherein the pseudotyped lentiviral vector contains an envelope protein selected from the group consisting of a vesicular stomatitis virus G (VSV-G) protein and a Moloney leukemia virus protein.

67. The method of claim 49, wherein the pseudotyped lentiviral vector is an HIV-1 vector.

68. The method of claim 67, wherein the pseudotyped HIV-1 vector comprises a VSV-G protein.

69. The method of claim 67, wherein the pseudotyped HIV-1 vector comprises an HIV-1 genome encoding nonfunctional HIV-1 env and nef gene products.

70. The method of claim 67, wherein the pseudotyped HIV-1 vector comprises an HIV-1 genome encoding nonfunctional HIV-1 env, nef and at least three accessory gene products.

71. The method of claim 70, wherein the pseudotyped HIV-1 vector comprises an HIV-1 genome encoding nonfunctional HIV-1 env, nef, vif, vpr and vpu gene products.

72. The method of claim 71, said pseudotyped HIV-1 vector comprising a VSV-G envelope protein.

73. The method of claim 66, wherein the pseudotyped HIV-1 vector contains a nucleotide sequence heterologous to HIV-1.

74. The method of claim 49, wherein the pseudotyped lentiviral vector is a non-HIV lentiviral vector.

75. A vector, comprising:

an envelope protein from a virus other than a lentivirus; and
a lentivirus genome encoding nonfunctional env and nef gene products.

76. The vector of claim 75, wherein the envelope protein is selected from the group consisting of a vesicular stomatitis virus-G (VSV-G) env protein and a Moloney leukemia virus env protein.

77. The vector of claim 75, wherein the lentivirus genome is a human immunodeficiency virus-1 genome.

78. The vector of claim 77, wherein the env protein is a VSV-G env protein.

79. The vector of claim 75, which contains a heterologous nucleic acid sequence.

80. The vector of claim 75, wherein a nucleic acid sequence encoding an env gene product or a nef gene product is deleted.

81. A vector, comprising:

an envelope protein from a virus other than a lentivirus; and
a lentivirus genome encoding nonfunctional env, nef, and at least three accessory protein gene products.

82. The vector of claim 81, wherein the envelope protein is selected from the group consisting of a vesicular stomatitis virus G (VSV-G) env protein and a Moloney leukemia virus env protein.

83. The vector of claim 81, wherein the lentivirus genome is a human immunodeficiency virus-1 (HIV-1) genome.

84. The vector of claim 81, wherein at least one nucleic acid sequence encoding a nonfunctional gene product is deleted.

85. The vector of claim 81, comprising:
an envelope protein selected from the group consisting of a vesicular stomatitis virus (VSV-G) protein and a Moloney leukemia virus protein; and
a lentivirus genome having deletions of nucleic acid sequences encoding env, nef, vif, vpr and vpu.

86. The vector of claim 85, wherein the lentivirus genome is a HIV-1 genome and the env protein is a VSV-G env protein.

87. The vector of claim 81, which contains a heterologous nucleic acid sequence.

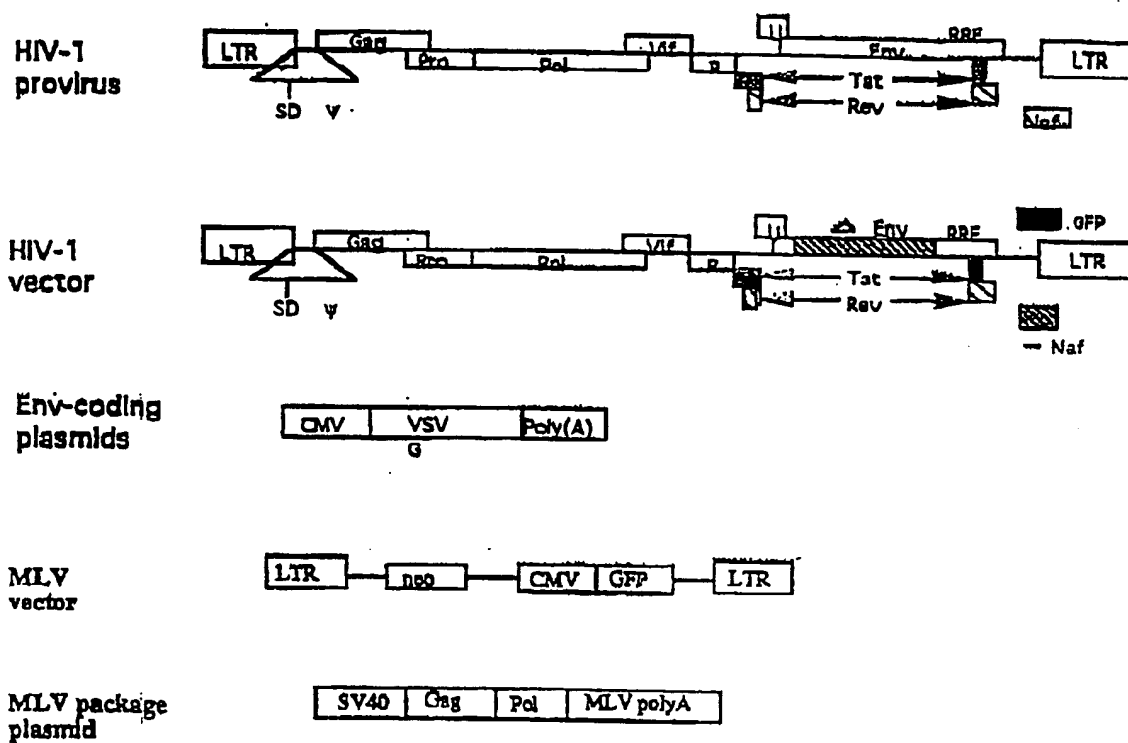


FIG. 1

Expression of GFP in CD34+ cells delivered by an HIV-1 vector

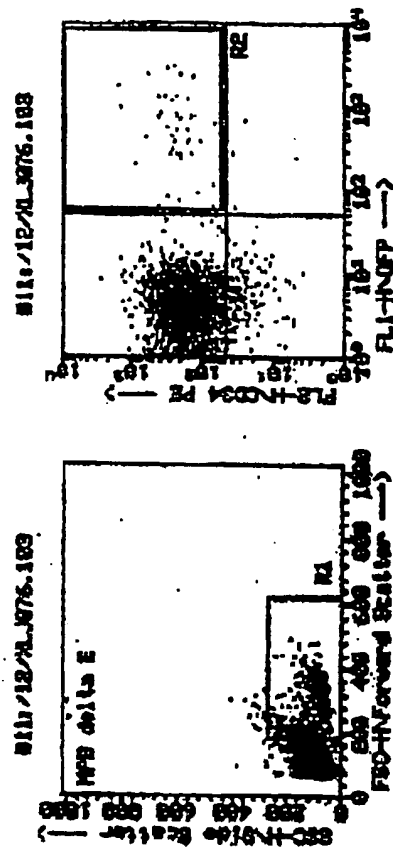


FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 00/23162

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/86 C12N5/10 A61K35/12 C12N15/62 A61K39/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 46083 A (KAN MITCHELL JUNE ;LI XINQIANG (US); UNIV CALIFORNIA (US); WONG ST) 22 October 1998 (1998-10-22) the whole document	1-87
X	LI X ET AL: "TRANSDUCTION OF CD34+ CELLS BY A VESICULAR STOMACH VIRUS PROTEIN G (VSV-G) PSEUDOTYPED HIV-1 VECTOR STABLE GENE EXPRESSION IN PROGENY CELLS, INCLUDING DENDRITIC CELLS" JOURNAL OF HUMAN VIROLOGY,US,PHILADELPHIA, PA, vol. 1, no. 5, July 1998 (1998-07), pages 346-352, XP000892959 ISSN: 1090-9508 the whole document	1-8,15, 19,75-87

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 00/23162

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	abstract page 1735, right-hand column, paragraph 2 -page 1736, left-hand column, paragraph 2; figure 2	1-74
X	SONG ES. ET AL.: "Antigen presentation in retroviral vector-mediated gene transfer in vivo." PROC NATL ACAD SCI U S A 1997 MAR 4;94(5):1943-8, XP002153788	75-87
Y	abstract	1-74
X	POESCHLA E ET AL: "DEVELOPMENT OF HIV VECTORS FOR ANTI-HIV GENE THERAPY" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 93, 1 October 1996 (1996-10-01), pages 11395-11399, XP000616290 ISSN: 0027-8424	75-87
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P,X	GRUBER A. ET AL.: "Dendritic cells transduced by multiply deleted HIV-1 vectors exhibit normal phenotypes and functions and elicit an HIV-specific cytotoxic T-lymphocyte response in vitro." BLOOD 2000 AUG 15;96(4):1327-33, XP000943555 the whole document	1-87

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/23162

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